APPLICATION

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TITLE: STREPTOCOCCUS PYOGENES VACCINE

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~~STREPTOCOCCUS PYOGENES VACCINE~~
Vaccines Containing Cysteine Protease And Methods To Protect Against Group A Streptococci

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of molecular bacteriology and infectious disease. More specifically, the present invention relates to a *Streptococcus pyogenes* vaccine useful for the prevention of a variety of human diseases.

Description of the Related Art

Streptococcus pyogenes is a Gram-positive bacterium that is the etiological agent of several diseases in humans, including pharyngitis and/or tonsillitis, skin infections (impetigo, erysipelas, and other forms of pyoderma), acute rheumatic fever (ARF), scarlet fever (SF), poststreptococcal glomerulonephritis (PSGN), and a toxic-shock-like syndrome (TSLS). On a global basis, ARF is the most common cause of pediatric heart disease. For example, it is estimated that in India more than six million school-aged children suffer from rheumatic heart disease. In the United States, "sore throat" is the third most common reason for physician office visits and *S. pyogenes* is recovered from about 30% of children with this complaint. There are about 25-35 million cases of streptococcal pharyngitis per year in the United States, responsible for about 1-2 billion dollars per year in health care costs. In recent years, an intercontinental increase in streptococcal disease frequency and severity has occurred for unknown reasons, although two variant pyrogenic exotoxin A molecules have been implicated. The amino acid residues characterizing the mutant SPEA molecules are located in an area of the toxin that, based on the

recently published three-dimensional crystal structure of the related enterotoxin B from *Staphylococcus aureus,* forms the T-cell receptor binding groove.

The continued great morbidity and mortality caused by *S. pyogenes* in developing nations, the significant health care financial burden attributable to Group A streptococci in the United States, and increasing levels of antibiotic resistance in this pathogen highlight the need for a fuller understanding of the molecular pathogenesis of streptococcal infection. Moreover, the recent disease increase underscores the lack of an efficacious vaccine, despite the repeated inclusion of *S. pyogenes* in lists of important human pathogens for which vaccines are needed.

S. pyogenes synthesizes an extracellular proenzyme zymogen of 371 amino acids (40,314 kDa) that can be transformed into an enzymatically active protease of 253 amino acids (27,588 kDa) by reduction and autocatalytic conversion. The zymogen contains one or more epitopes not associated with the truncated enzyme. Both the zymogen and active protease contain a single half-cysteine per molecule that is susceptible to sulfhydryl antagonists. In broth cultures, inactive precursor accumulates extracellularly during bacterial multiplication and reaches a maximum concentration at the end of logarithmic growth. Some strains yield up to 150 mg/liter of zymogen, and the molecule is a major extracellular protein. Thus, the streptococcal cysteine protease resembles many secreted bacterial extracellular protease virulence factors in having a specific signal peptide and a pro-sequence that is removed in an autocatalytic fashion to generate a fully active enzyme.

Protection against systemic streptococcal infection is thought to be due predominantly to type-specific opsonic anti-M protein IgG. As a consequence, immunoprophylaxis research has been conducted almost exclusively in the context of formulating an M protein vaccine. However, two major theoretical and practical problems have hindered this approach. First, more than 80 distinctive M protein types have been described based on serological and gene sequencing studies. The occurrence of this extensive array of serotypic variants means that either an effective

M protein vaccine must be very heterogenous in composition or that conserved protective M protein elements must be used. Formulation of a highly polyvalent vaccine has generated little enthusiasm, and a conserved pan-protective M protein fragment has yet to be identified. A second problem that has plagued M protein vaccine research is the observation that M proteins contain epitopes that cross-react with heart and other human tissue. This fact, in concert with the presumed autoimmune aspects of several streptococcal diseases, has also slowed M protein vaccine development.

The prior art remains deficient in the lack of an effective vaccine for the immune prophylactic prevention of Group A streptococcal infection. In addition, the prior art is deficient in the lack of an efficient method of immunizing a human against Group A streptococcal infections.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a vaccine against Group A streptococcal infection, comprising: a physiologically acceptable non-toxic vehicle containing a conserved cysteine protease, a natural or engineered non-proteolytic variant, or a peptide fragment of the protease.

In another embodiment of the present invention, there is provided method of immunizing mammals against Group A streptococcal infection, comprising: administering the vaccine of the present invention to said mammal in an amount sufficient to confer immunity to Group A streptococcal infection.

In yet another embodiment of the present invention, there is provided a method of immunizing mammals against Group A streptococcal infection, comprising: administering the vaccine containing a cysteine protease or a natural or engineered non-proteolytic variant and a streptococcal M protein to said mammal in an amount sufficient to confer immunity to Group A streptococcal infection.

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

Figure 1 shows purified streptococcal cysteine protease. Streptococcal cysteine protease was purified from strain MGAS 1719 and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1:2 μg of the purified protease; lane 2: molecular weight standards.

Figure 2A-2B show ~~Figure 2 shows~~ the cleavage of human interleukin-1β (IL-1β) precursor by streptococcal cysteine protease. Figure 2A shows the [^{35}S]methionine labeled pIL-1β (lane 1) synthesized in a rabbit reticulocyte lysate system and incubated with 250 ng of purified cysteine protease (lane 2) or boiled cysteine protease (lane 3) for 1 hour. A human pIL-1β Asp 116-> Ala 116 mutant was also cleaved by the cysteine protease (lane 4). Figure 2B shows the western immunoblot analysis of mature and recombinant pIL-1β cleavage products. Lane 1:mIL-1β alone; lane 2: mIL-1β plus the cysteine protease; lane 3: pIL-1β alone; lane 4: pIL-1β plus the cysteine protease. Incubations were conducted for 30 minutes at 37°C. The cleavage products were resolved by SDS-PAGE, transferred to nitrocellulose and probed with carboxy-terminal-specific monoclonal antibody. The ~18.5 kDa product was converted to the lower molecular weight form upon further incubation. The immunoreactive proteins of greater and less than ~33 kDa in lane 3 are produced in the fermentation process used to make pIL-1β.

Figure 3 shows the stimulation of nitric oxide (NO) synthase activity by the cysteine protease and pIL-1β in rat aortic smooth muscle cells. Cells were treated for 24 hours with either SFM (1), mature IL-1β (3 ng/ml) (2), cysteine protease (4 mg/ml) (3), pIL-1β (~200 ng/ml) (40< or cysteine protease plus pIL-1β(5). The

nitrite concentration in conditioned media samples from treated cells was determined by comparison with a sodium nitrite standard curve.

Figure 4 shows the cleavage of pIL-1β by allelic variants of streptococcal cysteine protease. Rabbit reticulocyte lysate containing [^{35}S]methionine-labeled pIL-1β (lane 1) was incubated with cysteine protease purified from strain MGAS 279 (lane 2) or MGAS 289 (lane 3) as described for Figure 2A.

Figures 5A-5C show ~~Figure 5 shows~~ the cleavage of purified extracellular matrix (ECM) proteins.

Figures 6A-6B show ~~Figure 6 shows~~ the induction of cytopathic effect and fibronectin cleavage in human umbilical vein endothelial cell (HUVEC) cultures.

Figure 7 shows the *spe*B production by *S. pyogenes* strains.

Figure 8 shows the processing sites, locations of amino acid variations found in the proteins made by the *spe*B2 and *spe*B4 alleles, and amino acids that are targets for mutation.

Figure 9 shows the generation of random mutations in *spe*B.

Figure 10 shows the mouse protection with rabbit anti-cysteine protease IgG. Mice were injected i.p. with 0.1 ml of 1mg rabbit antibody (IgG) in PBS (pH 7.4) raised against cysteine protease purified from strain MGAS 1719, which has *spe*B 7 allele and produce the SPE B 4 mature SPE B variant. The antibody was raised in rabbits against purified protease excised from SDS polyacrylamide gel. Control animals received either an equal amount of pre-immune antibody (0.1 ml containing 1 mg of IgG in PBS, pH 7.4) from the same rabbit used as the source for the anti-cysteine protease, or 0.1 ml of PBS. After 30 minutes, mice were injected i.p. with approximately 100 cfu of strain MGAS 315, which has the *spe*B3 allele and produces the SPE B1 mature SPE variant and is an ET2/m3 organism from a case of TSLS in (Musser et al. 1993). The time required for 50% mortality for the mice groups are as follows: PBS, 26 hours; pre-immune, 25 hours; anti-cysteine protease, 55 hours.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a vaccine against Group A streptococcal infection, comprising: a physiologically acceptable non-toxic vehicle containing a conserved cysteine protease or non-proteolytic variant or conserved peptide. Generally, the cysteine protease may be any that provokes an immune response, with consequent immunity to, Group A streptococcal infection. Preferably, the cysteine protease is a translated portion of the SPE B gene or fragments or derivatives thereof. Alternatively, the cysteine protease is a synthetic peptide.

Generally, the vaccine of the present invention may be useful in the prevention of any Group A streptococcal infections. Representative examples of Group A streptococcal infections include pharyngitis, tonsillitis, skin infections such as impetigo, erysipelas and other form of pyoderma, scarlet fever, acute rheumatic fever, scarlet fever, post-streptococcal glomerulonephritis, sepsis and toxic-shock-like syndrome.

In one embodiment of the present invention, the efficacy of the vaccine may be enhanced by the addition of a streptococcal M protein antigen. In this vaccine, the conserved domain of the streptococcal M protein is combined with the cysteine protease.

The present invention also provides a method of immunizing mammals against Group A streptococcal infection, comprising: administering the vaccine of claim 1 to a human in an amount sufficient to confer immunity to Group A streptococcal infection.

Generally, the vaccine may be administered in any dose or amount that produces a satisfactory immune response and that will confer immunity to the mammal from Group A streptococcal infections. Determination of an appropriate dose for the vaccine of the present invention is within the ordinary skill of those in this art.

The vaccine may be given by a number of different routes of administration. Preferably, the vaccine is given by parenteral administration. For example, the vaccine may be given by subcutaneous administration or intramuscular administration. Alternatively, the vaccine is administered orally.

A person having ordinary skill in this art will readily recognize that the amounts of cysteine protease or Group M streptococcal protein may be modified to achieve the greatest therapeutic benefit. For example, the vaccine may need to be administered in multiple doses.

The present invention also provides a method of immunizing mammals against Group A streptococcal infection, comprising: administering the vaccine of the present ~~invention~~claim 5 to a human in an amount sufficient to confer immunity to Group A streptococcal infection.

The following examples are given for the purpose of illustrating various embodiments of the methods of the present invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Bacterial Isolates

Table 1 shows the 68 strains of *S. pyogenes* studied. MGAS 1719 is identical to strain B220, the designation assigned by Dr. R. Lancefield to strain 5797. The strain expresses type 8 T antigen but is serologically nontypeable for M protein.

Table 1 Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
1	M1	19	pharyngitis	USA - 1980s	*speB2*	-	I
	M1	166	TSLS	USA - 1980s	*speB2*		
	M1	285	SID	USA - 1980s	*speB2*		
	M1	326	TSLS	USA - 1980s	*speB2*		
	M1	480	invasive	Yugoslavia - 1990s	*speB2*		
	M1	579	cellulitis	Canada - 1980s	*speB2*		
	M1	1253	scarlet fever	UK - 1920s	*speB2*		
2	M3	75	pharyngitis	USA - 1980s	*speB3*	-	I
	M3	157	TSLS	USA - 1980s	*speB3*		
	M3	315	TSLS	USA - 1980s	*speB3*		
	M3	1251	scarlet fever	USA - 1920s	*speB3*		
34	M14	660	unknown	Egypt - 1971	*speB8*	-	I

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
35	M46	1222	ARF	USA - 1953	*speB2*	-	I
36	M76	1832	unknown	unknown	*speB33*	+	
21	M12	282	SID	USA - 1980s	*speB1*	-	I
37	M1	789	NP	USA - 1946	*speB5*	-	I
38	M41	1841	unknown	unknown	*speB29*	-	I
39	M33	807	blood	USA - 1969	*speB15*	-	I
40	PT5757	1871	unknown	unknown	*speB34*		

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
40	PT4854	1893	unknown	unknown	*speB3*		
42	M8	429	unknown	unknown	*speB7*	-	I
43	T8	1719	unknown	unknown	*speB7*		
44	M28	587	scarlet fever	Canada - 1980s	*speB18*	-	II
10	T28	289	SID	USA - 1980s	*speB11*		
45	M24	684	ARF	USA - 1964	*speB12*	-	I
45	M19	1294	ARF	USA - unknown	*speB13*	-	I

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
46	M29	694	unknown	Egypt - 1971	*speB16*	-	I
46	M44	1226	ARF	USA - 1950s	*speB22*	+	II
46	M31	427	unknown	unknown	*speB16*	-	I
47	M30	366	unknown	USA - 1940s	*speB13*	-	I
26	NT	262	invasive	USA - 1980s	*speB6*		
16	M66	168	invasive	USA - 1980s	*speB25*	+	II
15	M73	302	invasive	USA - 1980s	*speB17*		
14	M4	321	TSLS	USA - 1980s	*speB5*	-	II

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
48	M59	1882	unknown	unknown	*speB37*	+	I
49	M5	1289	ARF	USA - 1953	*speB14*	-	I
20	M18	156	TSLS	USA - 1980s	*speB1*	-	I
	M18	300	invasive	USA - 1980s	*speB1*		
27	M6	303	invasive	USA - 1980s	*speB6*	-	I
50	M43	1842	unknown	unknown	*speB36*	-	I
51	M17	1233	ARF	USA - 1944	*speB21*	-	I
52	M23	1901	unknown	unknown	*speB35*	-	I

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
53	M49	719	impetigo	Trinidad - 1976	*speB10*	+	II
54	M15	1898	unknown	unknown	*speB32*	+	II
55	M25	686	wound	USA - 1969	*speB9*	+	II
24	M12	590	SID	Canada - 1980s	*speB3*	-	I
24	M22	162	SID	USA - 1980s	*speB28*	+	II
56	M9	800	impetigo	USA - 1964	*speB20*	+	II
57	M56	1864	unknown	unknown	*speB3*	-	I
58	M10	1896	unknown	unknown	*speB39*		

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
59	NT	1991	blood	USA - 1993	*speB26*		
	M75	758	ARF	USA - 1986	*speB26*		
	M75	1911	unknown	unknown	*speB26*		
5	NT	165	TSLS	USA - 1980s	*speB4*		
4	M2	327	TSLS	USA - 1980s	*speB3*	+	II
60	M9	796	unknown	USA - 1970	*speB19*	+	II
61	M11	650	NP	Trinidad - 1972	*speB23*	+	II
	M11	2075	invasive	Canada - 1980s	*speB* null	+	II
62	M62	1883	unknown	unknown	*speB17*	+	II

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
63	M13	659	unknown	Egypt - 1971	*speB24*	+	II
64	PT4931	1870	unknown	unknown	*speB31*		
65	TR2612	1872	unknown	unknown	*speB38*		
66	M27	1838	unknown	unknown	*speB30*	-	I
67	NT	2017	pharyngitis	USA - 1991	*speB24*		
67	NT	2018	pharyngitis	USA - 1992	*speB24*		
68	NT	1990	pharyngitis	USA - 1993	*speB27*		
69	TR2233	1914A	unkown	unknown	*speB26*		

Table 1, continued. Properties of 68 *S. pyogenes* strains representing 50 ETs[a]

ET	Serotype[b]	MGAS no.[c]	Disease or site[d]	Country and year	*speB* allele	opacity factor phenotype	M protein class
32	NT	317	invasive	USA - 1980s	*speB3*		

[a]ET, electrophoretic type.

[b]NT, nontypeable for M protein serotype.

[c]MGAS, Musser group A *Streptococcus* referencenumber. Strain sources and original designations are as follows: J. C. Huang, Laboratory Centre for Disease Control, Ottawa, Canada, MGAS 579 (11111), 587 (9378), 590 (11078), 2075 (DC11435); J. E. Peters, Wilford Hall Medical Center, San Antonio, Texas, MGAS 1991 (BB6672-3), 1990 (BA9812-4), P. M. Schlievert, University of Minnesota, Minneapolis, Minnesota, MGAS 1253 (119/6. also known as SF130/13), MGAS 1251 (C203S), 166 (Reineke), 285 (195), 325 (89.5.5612), 157 (Zinke), 315 (Soldier 1), 282 (192), 289 (199), 262 (Cal 17), 168 (Reinary), 302 (Lambert), 321 (Weckmuller), 156 (Wilson), 300 (Kluss), 303 (Lundeen), 162 (Cygan), 165 (Wicks), 317 (Timmers); E. L. Kaplan, University of Minnesota, MGAS 480 (90-441); M. A. Kehoe, University of Newcastle upon Tyne, Newcastle upon Tyne, England, MGAS 1841 (M41), 1871 (PT5757), 1893 (PT4854), 1882 (M59), 1842 (M43), 1901 (M23), 1898 (M15), 1864 (M56), 1896 (M10), 1911 (M75), 1881 (M62), 1870 (PT4931), 1872 (TR2612), 1838 (M27), 1914A (TR2233);

D. LeBlanc, University of Texas Health Science Center at San Antonio, Texas, MGAS 1222 (Cole 36XA87), 1226 (Cole 40XF1), 1233 (Cole 45XA9), K. H. Johnston, Louisiana State University Medical Center, New Orleans, Louisiana, MGAS 1719 (B220); D. E. Bessen, Yale University, New Haven, Coinnecticut, MGAS 1832 (CS110), 1294 (1RP232), 1289 (1RP144); S. K. Hollingshead, Department of Microbiology, University of Alabama School of Medicine, Birmingham Alabama, MGAS 660 (D469), 789 (1GL100), 807 (D323), 429 (C256/86/3), 684 (1RP284), 694 (D470), 427 (J137/69/1), 366 (AGL130), 719 (D938), 686 (D316), 800 (A724), 758 (86-809), 796 (D339), 650 (D691), 659 (D474).

All other strains are from the collection of J.M.M.

[d]TSLS, toxic-shock-like syndrome; SID, severe invasive disease; ARF, acute rheumatic fever; NP, nasopharynx.

EXAMPLE 2

Purification of the Cysteine Protease

Bacteria were grown overnight at 37°C in 5% CO_2 on brain-heart infusion (BHI) agar. The overnight culture was used to inoculate 200 ml of BHI liquid medium, and the culture was incubated for 12-14 hours at 37°C in 5% CO_2. A 50 ml aliquot of the overnight growth was added to 2 liters of chemically defined medium (JRH Bioscience, Lenexa, KS), pH 6.0, and the culture was incubated at 37°C in 5% CO_2. The broth was maintained at pH 5.5 - 6.0 by the addition of sterile sodium bicarbonate (10% w/v). After 8-9 hours, the cells were removed by centrifugation and the supernatant was concentrated to 250 ml by passage through a 10 kDa cutoff spiral ultrafiltration cartridge (Amicon). Buffer exchange (> 99%) by diafiltration was conducted with 1.5 liters of 20% ethanol - 20 mM Tris-HCl, pH 7.0 (buffer A) at 4°C, and the material was stored overnight at 4°C. The diafiltered solution was passed through a matrix gel red A (Amicon) column (1.5 cm x 15 cm) equilibrated with buffer A. The column was washed with buffer A until the adsorption (280 nm) returned to baseline, and the protein was eluted with buffer A containing 2M NaCl. The eluted material was collected as one fraction, and concentrated to 3 ml by ultrafiltration (Centriprep 10, Amicon), and the buffer was exchanged with PBS, pH 7.2, by gel-filtration chromatography (Bio-Rad).

Aminoterminal sequencing of the purified protein derived from dye-ligand affinity chromatography (Figure 1) reveals a sequence of -QPVVKSLLDSK-, (SEQ ID NO:1) corresponding to amino acids 146 - 156, thereby confirming the identity of the purified material as the truncated mature active form of streptococcal cysteine proteinase. The enzyme is stable for at least several months at -20°C. Three distinctive SPEB allelic variants (identified by sequencing studies) have been purified. The zymogen form can be purified with a closely similar protocol, except cysteine is omitted from the medium and the culture is incubated in the absence of

supplemental CO_2.

The published amino acid sequence for cysteine proteinase, including the configuration of the presumed active site, is incorrect. The predicted amino acid sequence encoded by this sequence is not cognate with the published cysteine protease sequence. Instead, the nucleotide sequence resembles, but is distinct from, the allele described by Hauser and Schlievert. However, the configuration of amino acids around the active cysteine residue is identical in strain B220 and all strains characterized thus far. Therefore, the proposition of Hauser and Schlievert (1990) that the lack of protease activity associated with SPEB purified from their M12 strain 86-858 is a consequence of the difference in amino acid sequence around the Cys residue is incorrect.

EXAMPLE 3

Cleavage of pIL-1β by Streptococcal Cysteine Protease

An assay employing radiolabeled pIL-1β made in a rabbit reticulocyte transcription-translation system was used. The cysteine protease produced a cleavage product of approximately 18 kDa, a size very similar to the apparent molecular weight of mIL-1β (Figure 2A). Western blot analysis of the cleavage products generated from recombinant pIL-1β made in *E. coli* confirmed this result (Figure 2B).

The cysteine protease cleaved a human pIL-1β mutant (Asp 116-> Ala 116, creating an Ala 116 - Ala 117 linkage) that is not degraded by ICE. As observed with wild type pIL-1β, cysteine protease cleaved the mutant substrate to form a product with an apparent molecular weight of ~18 kDa (Figure 2A). Thus, the primary cleavage site for the cysteine protease was not the ICE proteolytic site.

To determine exactly where the cysteine protease cleaved pIL-1, the

aminoterminal 10 amino acid residues of the ~18 kDa product made by degradation of recombinant pIL-1β was sequenced. The cysteine protease cleaved pIL-1β between His 115 - Asp 116 to create a molecule one amino acid residue longer than mIL-1β.

EXAMPLE 4

Normal Biological Activity of the Mature IL-1β Cleavage Product

Because a highly active form of mIL-1β with Asp-116 at the aminoterminus was described in the course of characterization of a metalloprotease found in human peripheral blood mononuclear cells, cysteine protease was processing inactive pIL-1β to biologically active IL-1β. Mature IL-1β is a potent inducer of nitric oxide synthase (NOS) activity in vascular smooth muscle cells (SMC). Cysteine protease was added in the presence or absence of pIL-1β to confluent cultures of SMC and NOS activity was assayed by measuring nitrite anion levels in the medium after 24 hours. Neither cysteine protease nor pIL-1β alone produced a significant increase in nitrite levels. In contrast, addition of cysteine protease and pIl-1β together caused approximately a 60-fold increase in nitrite accumulation (Figure 3).

IL-1β generated by cysteine protease cleavage of pIL-1β was also found to be active in the A375 cell line assay. In an assay in which approximately 500 ng/ml of intact pIL-1β was inactive, a cysteine protease digest of this material yielded 6.1 x 10^4 units/ml of activity; 500 ng/ml of authentic IL-1β corresponded to 1.1 x 10^5 units in this assay.

EXAMPLE 5

Cleavage Activity of Variant Cysteine Protease Enzymes

Two additional naturally occurring cysteine protease allelic variants

(SPE B2 and SPE B11) also produced an IL-1β fragment with an apparent molecular weight identical to that made by SPE B7 purified from MGAS 1719 (Figure 4).

EXAMPLE 6

Sequencing of speB

The *speB* gene was amplified by the polymerase chain reaction (PCR), with synthetic oligonucleotide . The DNA fragment studied (1.437 bp) represents the entire coding region (1,197 bp) and 160 bp of upstream and 80 bp of downstream sequence. For about one-third of the strains, single-stranded DNA was prepared by the lambda exonuclease method and sequenced in both orientations with Sequenase version 2.0. Variant alleles were sequenced again to confirm the nucleotide changes. Basically, the sequencing of the cysteine protease structural gene was as follows. The cysteine protease structural gene was amplified by the polymerase chain reaction (PCR), with synthetic oligonucleotides. The oligonucleotide primers used to amplify *speB* and flanking regions were as follows:

SPEB-X, 5' - GTTGTCAGTGTCAACTAACCGT = 3'; and (SEQ ID NO:01)

SPEB-2, 5' = ATCTGTGTCTGATGGATAGCTT - 3'. (SEQ ID NO:02)

The following four oligonucleotides were used as internal sequencing primers:

SPEB-1, 5' - CTTTCTGGCTCTAATATGTATGT - 3'; (SEQ ID NO:04)

SPEB-3, 5' - GTTATTGAAAAAGTAAAACC - 3'; (SEQ ID NO:05)

SPEB-4, 5' - TTTTCAATAACAGGTGTCAA - 3'; and (SEQ ID NO:06)

SPEB-Y, 5' - TCTCCTGAAACGATAACAAA - 3'. (SEQ ID NO:07)

PCR amplification of 1 μl of chromosomal DNA was performed in 100 μl of a mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM each of dATP, dCTP, dGTP, and dTTP, 200 nM each of SPEB-X and SPEB-2, and 2.5 units of *AmpliTaq* DNA polymerase. The thermocycling parameters were denaturation at 94°C for 1 minute, annealing at 55°C for 2

minutes, and extension at 72°C for 2.5 minutes for a total of 30 cycles. A final extension at 72°C for 15 minutes was used.

The DNA fragment (1,437 bp) represents the entire coding region (1,197 bp) and 160 bp of upstream and 80 bp of downstream sequence. For about one-third of the strains, single-stranded DNA was prepared by the lambda exonuclease method and sequenced in both orientations with Sequencase version 2.0. Variant alleles were sequenced again to confirm the nucleotide changes.

The protease gene in approximately two-thirds of the strains was characterized by automated DNA sequencing with an Applied Biosystems, Inc., Model 373A instrument. For the automated approach, the gene was amplified with PCR (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 2.5 units of *Taq* polymerase; 20 picomoles of each primer; 1 μL of chromosomal DNA template), with the following thermocycler parameters: denaturation at 94°C for 4 minutes, 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 2 minutes, extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. The unincorporated nucleotides and primers were removed by filtration through Microcon 100 microconcentrators (Amicon Inc., MA). Sequencing reactions with the *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., CA) were performed with 7 μL of PCR amplified DNA as template and 3.2 picomoles of primer. The unincorporated dye terminators and primers were separated from the extension products by spin column purification (Centri-Sep, Princeton Separations, Inc., NJ). The sample was dried in a vacuum centrifuge. Prior to gel loading, the sample was resuspended in 4 μL of sample loading buffer (5:1 deionized formamide; 50 mM EDTA, pH 8.0) and heat denatured for 2 minutes at 90°C. The data were assembled and edited with EDITSEQ, ALIGN, and SEQMAN programs (DNASTAR, WI).

EXAMPLE 7

Estimates of Genetic Relationships Among Clones

Methods of estimating genetic relationships among *S. pyogenes* clones by multilocus enzyme electrophoresis were as described by Musser et al., **Proc. Natl. Acad. Sci. USA** 88:2668-72 (1991). Thirty-six ETs not identified previously were arbitrarily numbered ET 34 - ET 53.

EXAMPLE 8

speB Allele in Strain B220 (Elliott 5797)

The speB gene (speB7) in strain MGAs 1719 does not encode a protein with the amino acid sequence presented previously. There are discrepancies between the protein sequence from strain B220 and a *speB* allele (herein designated *speB1*) in a serotype M12 strain (86-858).

EXAMPLE 9

speB Alleles and Disease Type

The present invention demonstrates that streptococcal clones with the same *speB* allele, and *speB* allele - M protein combination are associated with several different diseases. For example, strains of ET 1 - M1 - *speB2* were cultured from patients with pharyngitis, scarlet fever, cellulitis, and TSLS; and ET 2 - M3 - *speB3* organisms were recovered from cases of pharyngitis, scarlet fever, and TSLS. Similarly, strains cultured from individuals with acute rheumatic fever had six distinct *speB* alleles. Hence, there was no apparent preferential association of speB allele and disease type.

The identification of the *speB* allele in a strain (MGAS 789) recovered in the 1940s expressing M1 protein, but assigned to ET 36 rather than ET 1 like contemporary M1 strains suggests that variation in *speB* allele - multilocus enzyme genotype - M protein associations made by a contributing factor in temporal changes in streptococcal disease frequency and severity.

EXAMPLE 10

speB Variation, M Protein Class, Opacity Factor Phenotype, and vir Regulon Architecture

The present invention found no compelling evidence for an analogous differentiation of *speB* allelic variants. Strains assigned to either of two distinct classes based on reactivity with a panel of monoclonal antibodies to M protein did not have consistent sequence differences, and in several instances the identical *speB* allele was found in strains of two M protein classes. For example, the *speB3* allele occurred in strains of both class I (M3 and M12) and class II (M2), and similarly, the *speB5* allele was identified in strains expressing M1 and M4 assigned to class I and class II, respectively (Table I). Similarly, there was no simple congruent relationship between *speB* allele and *vir* regulon architecture or opacity factor phenotype. M2, M3, and M12 strains all had the *speB3* allele, but, M3 and M123 are opacity factor-negative and M2 is opacity factor-positive. The lack of a significant correlation between M serotype class and *speB* phylogeny could also be caused by relatively frequent lateral transfer events involving part or all of the *emm* and *speB* genes.

EXAMPLE 11

Cleavage of purified extracellular matrix (ECM) proteins

Streptococcal cysteine protease rapidly degrades purified vitronectin (VN)

(Figure 5). After five minutes of protease incubation with VN, degradation products could not be identified by either Coomassie blue staining or immunoblotting with polyclonal anti-VN antibodies. Similarly, the streptococcal protease cleaved fibronectin (FN) immediately, as shown by the rapid appearance of lower molecular weight products (Figure 5). However, in contrast to VN degradation, FN cleavage apparently occurred at a limited number of specific sites (Figure 5) of manuscript). Incubation of FN with the protease for up to 12 hours did not result in formation of additional degradation products.

No significant cleavage of human laminin (LN) was observed under the experimental conditions assayed (Figure 5), or when 10 μg of protease and 2 μg of LN substrate were used.

EXAMPLE 12

Induction of cytopathic effect and fibronectin cleavage in human umbilical vein endothelial cell (HUVEC) cultures

Because patients with invasive *S. pyogenes* episodes frequently have bacterial sepsis with endothelial cell damage, the ability of the streptococcal cysteine protease to cleave FN directly from HUVECs grown in culture was examined. Western immunoblot analysis of cells in the absence of protease, or treated with boiled protease for up to 8 hours, showed no detectable FN degradation of (Figure 6). In contrast, cells incubated with as little as 6 μg/ml of streptococcal protease per well for 2 hours retained only a small fraction of intact native FN. Thus, the streptococcal protease cleaves FN in a dose and time dependent manner in the complex environment of cells growing in tissue culture.

Interestingly, treatment of HUVECs with the streptococcal protease rapidly induced striking cytopathic effects (Figure 6). By 3 hours after protease addition, zones of clearing occurred in the cell monolayer. This effect was followed

by loss of cell adherence to the matrix and ablation of the characteristic cobblestone morphology. FN cleavage was detectable by immunoblot analysis prior to the onset of cytopathic effect. Bands that correspond to native human VN in either the solubilized control or treated HUVECs by western immunoblot analysis were not seen, presumably due to low level or lack of VN expression by these cells.

EXAMPLE 13

Cysteine protease production by *S. pyogenes* strains

Virtually all clinical isolates of Group A streptococci produce SPE B/cysteine protease and patients infected with Group A streptococci develop antiproteinase antibodies. Immunoblot analysis of culture supernatants was used to assess production of SPE B/streptococcal protease by strains of *S. pyogenes,* and one naturally occurring serotype M11 isolate (MGAS 2075) reported to lack *speB.* With the exception of the three strains, all 64 other isolates examined produced cysteine protease, a result consistent with the notion that virtually all *S. pyogenes* strains express the molecule extracellularly (Figure 7 and Table I). The three strains had alleles *speB3, speB13,* and *speB16,* but other isolates with these same alleles produced the protease. Therefore, all 39 *speB* alleles can be expressed by Group A streptococcal strains under appropriate conditions.

EXAMPLE 14

Specific antisera raised against the active cysteine protease

Purified protease (100 μg) mixed with Freund's complete adjuvant was injected subcutaneously at multiple sites into two rabbits. Subsequent immunizations with the purified protease mixed with Freund's incomplete adjuvant were conducted at bi-weekly intervals for a total of five injections. Serum was collected and

immunoglobulin purified by FPLC with a protein G-Sepharose column (Pharmacia). Western immunoblot analysis revealed the presence of specific anti-protease reactivity in the post-immunization samples but not in the pre-immunization sera. These rabbits are being maintained and bled at regular intervals to collect large quantities of specific antiserum.

EXAMPLE 15

Mouse monoclonal antibodies against the purified mature cysteine protease

Monoclonal antibodies against the purified mature cysteine protease were prepared. A dose of 10 μg of SDS-PAGE purified mature protease has been injected a total of five times intraperitoneally into five mice (*Balb/c* background). Western immunoblot analysis has demonstrated that all mice have serocoverted. The spleens were harvested and fusions performed by standard protocols. Characterization of protease-specific monoclonal antibodies is by standard procedures.

EXAMPLE 16

Measurement of antibody levels

An ELISA has been developed to measure antibody levels against the cysteine protease. Briefly, 10 μg of protease in carbonate-bicarbonate buffer (pH 9.6) was added to each well of a 96-well microtiter plate and incubated overnight at 4°C. The wells were rinsed three times with washing buffer [PBS (pH 7.4) - Tween 20 (0.05%)] and blocked with 200 μL of 0.5% BSA in PBS, pH 7.4, for 2 hours at 37°C. After washing, the wells were charged with 100 μL of a serial dilution of test antisera (1:100 through 1:1600 of rabbit serum). The plate was incubated for 1 hour at 37°C, washed again, and 100 μL of a 1:5000 dilution of

extravidin-alkaline phosphatase was added to each of the test wells and incubated at 37°C for 30 minutes. After washing, 100 μL of alkaline phosphatase substrate (pNPP) was added to each well and reacted for 1 hour at room temperature. The O.D. (405 nm) was read with a microtiter plate reader.

EXAMPLE 17

Immunodot-blot assay for cysteine protease expression

A dot-blot assay was developed that detects as little as 1 nanogram of cysteine protease. Briefly, test material (usually protein precipitates of culture supernatants from bacteria grown in chemically defined medium) was spotted onto a nylon membrane, and unabsorbed sites were blocked by incubation with 0.5% blocking agent (Amersham) for 1 hour at room temperature. The membrane was rinsed with PBS (pH 7.4) - Tween 20 (0.05%) and incubated for 30 minutes with purified polyclonal rabbit antiserum (1:500 dilution) directed against the cysteine protease. The membrane was rinsed with PBS, a secondary antibody (goat anti-rabbit-HRP conjugate, 1:2000 dilution) was added and incubated for 30 minutes at room temperature. The blot was visualized with chemiluminescence (ECL developing reagents, Amersham). With this technique, many isolates previously reported to lack SPEB production based on less sensitive conventional immunologic assays express the cysteine protease.

EXAMPLE 18

Antibody directed against cysteine protease

The immunoprophylactic protection of cysteine protease is seen by the use of two models. First, the intranasal immunization model is used as developed by Bessen and Fischetti (1988) to evaluate the effect of cysteine protease immunization

on mucosal colonization by *S. pyogenes.* Second, a mouse cutaneous infection model (Bunce et al., 1992) is used against a subcutaneous bacterial challenge. Briefly, the animals are injected with protease s.c. on the flank and observed daily, including weight measurements. Abscess volumes and area of dermonecrosis is calculated and lesion size curves are determined.

EXAMPLE 19

Preparation of Synthetic Peptides of Cysteine Protease

Synthetic peptides based on cysteine protease may also be used as immunogens in the preparation of a vaccine against Group A streptococcal infections. Several synthetic peptides are selected based on the location of allelic variation and conservation and the cysteine protease antigenic index generated with a Jameson-Wolf plot. First, each of the following three peptides are used. These peptides correspond to the variable region (amino acids 308 to 317) in mature streptococcal cysteine protease containing two of the six major calculated antigenic peaks.

Peptide 1: H-Q-I-N-R-S(308)-D-F-S-K-Q-D-W-E-A(317)-Q-I-D-K-E (SEQ ID NO:8)

Peptide 2: H-Q-I-N-G(308)-D-F-S-K-Q-D-W-E-A(317)-Q-I-D-K-E (SEQ ID NO:09)

Peptide 3: H-Q-I-N-S(308)-D-F-S-K-Q-D-W-E-A(317)-Q-I-D-K-E (SEQ ID NO:10)

Subsequently, each of the following four peptide, which correspond to four invariant calculated antigenic peaks are used for immunization.

Peptide 4: P(171)-V-I-E-K-V-K-P-G-E-Q-S-F-V-G-Q (SEQ ID NO: 11)

Peptide 5: Y(203)-H-N-Y-P-N-K-G-L-K-D-Y-T-Y-T-L (SEQ ID NO:12)

Peptide 6: P(247)-T-Y-S-G-R-E-S-N-V-Q-K-M-A-I (SEQ ID NO: 13)

Peptide 7: I(344)-D-G-A-D-G-R-N-F-Y-H (SEQ ID NO: 14)

Naturally occurring variant zymogens and cysteine protease display unique linear B-cell epitopes.

Overlapping 10-mer peptides are used which overlap 2 amino acid residues with the previous one in the consecutive primary sequence corresponding to 371 amino acids of the mature cysteine protease zymogen (translated product minus leader sequence). Synthetic 10-mers corresponding to the 10 variant amino acid residues will also be used. The variant amino acids are positioned in the middle of the 10-mer. For example, if the sequence of a 10-mer corresponding to one region of the SPEB1 variant is position 304-QINRSDFSKQ-313, then 304-QINRGDFSKQ -313 is also examined, a 10-mer that incorporates a variant amino acid found in the SPEB2 variant. Once the 10-mer peptides are synthesized, an ELISA is used to examine the reactivity of all peptides with the following materials: (i) rabbit polyclonal hyperimmune antiserum made against purified cysteine protease (positive control), (ii) rabbit pre-immune serum (negative control), (iii) our panel of 28 murine monoclonal antibodies raised against purified cysteine protease, (iv) acute and convalescent sera obtained from 20 patients with necrotizing fascitis and/or TSLS in Canada (obtained from D. Low, Mount Sinai Hospital, Ontario, Canada), 5 USA patients with TSLS characterized by extensive soft tissue destruction (obtained from D. Stevens, V.A. Hospital, Boise, Idaho), and 5 patients with ARF (obtained from A. Bisno, University of Miami Medical School). The great majority of the synthetic peptides usually are not reactive with each sera and there are a large number of internal redundant negative control peptides. Sera dilutions are used in these assays (1:1000 for hyperimmune rabbit antiserum, 1:500 for human serum, and 1:5 - 1:10 for MAb culture supernatants).

To determine the linear B-cell epitopes, for each sera and MAb tested, one plots OD_{405} versus 10-mer peptide number. The linear B-cell epitopes are displayed as a peak in the OD_{405} values. In general, a peak is composed of several contiguous overlapping peptides, and the 10-mer peptide with the highest OD value are defined as the parent peptide.

The pro region contains at least one unique linear B cell epitope. The

same linear B cell epitopes will most likely be recognized by all 15 human convalescent sera specimens.

EXAMPLE 20

Creation of mutant speB proteins

Figure 8 shows the processing sites, locations of amino acid variations found in the proteins made by the *speB2* and *speB4* alleles, and amino acids that are targets for mutation. Both site-directed and random mutagenesis schemes are employed to identify residues that disrupt cysteine protease function and zymogen processing, and to map regions that constitute antigenic domains of the protein. Targets for functional amino acid replacement are based on biochemical analysis of cysteine protease (Tai, et al., 1976) and by analogy with similar residues ins the eukaryotic cysteine protease, papain (Kamphuis, et al., 1984).

To create a stable zymogen to facilitate crystallographic studies and generate enzymatically deficient or inactive protease for structure-function studies, mutant forms of the cysteine protease protein are made and characterized. A targeted mutagenesis scheme creates changes that: (i) disrupt protease activity; (ii) prevent zymogen processing; (iii) prevent substrate binding; and (iv) alter immunoreactivity. Amino acids are changed to structurally neutral alanine. A mutant protein that lacks protease activity, but which retains antigenicity, is generated by mutagenesis of the single cysteine residue (Cys-192->Ala-192) at the catalytic site of the molecule. Also, His-340 and Gin-185 and Asn-356 are mutagenized. These three changes are epistatic to the Cys-192 mutation, but may alone exhibit altered activity. Trp-357, thought to be involved in substrate binding and similarly positioned within papain, is also be targeted. A stable zymogen precursor is also created by mutating residues surrounding the protease cleavage site at Lys-145. In addition, mutagenesis of Cys-192 may prevent autoproteolysis, as

occurred for a Cys->Ser mutant of papain, the prototype cysteine protease. Other mutagenesis targets include a putative nucleotide binding domain (GVGKVG) and a potential collagen docking region [$(GXX)_3$] within carboxy terminal portion of the protein. Site-directed mutagenesis is used, by the charged-to-alanine-scanning method, to substitute positively and negatively charged amino acids (often involved in recognition and activity) with alanine. Many of the charged residues (14 lysine, 7 arginine, 12 aspartate, and 7 glutamate residues in the mature peptide) are expected to lie on the surface of the cysteine protease structure, and some are expected to define epitopes on the molecule. In particular, a region of charged amino acids, from 307 to 321 (8/15 charged), is examined; this region includes the site of *speB2* and *speB4* amino acid substitutions. Residues in antigenic regions identified in the epitope mapping studies are also mutated.

First, the *speB* gene is amplified from an ET1/M1 *S. pyogenes* strain, with PCR, and the product is cloned into a multicopy filamid vector such as pBluescript (Stratagene, La Jolla, CA). This vector is chosen because it carries the regulated *lac* promoter and can be replicated as a single-stranded molecule for site-directed mutagenesis. Cloning is designed to place the promoter, ribosome binding site and *speB* reading frame 3' to the inducible *lac* promoter on the vector so that the protein can be conditionally over-expressed in *E. coli* when the *lac* inducer, IPTG, is added. The *speB* promoter is included requiring expression in *S. pyogenes.* Whole cell extracts and periplasmic shockates of *E. coli* cells carrying this primary *speB* clone are examined for the presence of the cysteine protease protein by SDS-PAGE and by Western blotting with anti-cysteine protease antibody. The resulting plasmid is the target for mutagenesis.

Oligonucleotide-directed mutations, such as substitutions, deletions and small insertions, are created on uracil-containing single-stranded templates by the method of Kunkel (Kunkel, 1985). When possible, mutagenic primers are designed to incorporate a unique restriction site into the *speB* gene for mapping and mutant

selection. Both single and multiple alanine substitutions are created at the residues indicated above. Once residues critical to function are identified, small regions surrounding them are deleted or substituted, by using the same methods, to further characterize the region and to preclude reversion. When crystallographic data is available, additional amino acids are mutated.

A random mutagenesis scheme is also employed. Variant proteins created by this method are most useful for epitope screening, although molecules with altered kinetics and substrate recognition may also be recovered. Regions of the *speB* sequence are randomized with mixed oligonucleotides in the primed-mutagenesis protocol, or short, in-frame deletions within the gene are created with a modification of the DNAse 1-linker insertion/deletion protocol of Palzkill and Bostein (Palzkill and Bostein, 1991). Here, synthetic "excision linkers" are first ligated to randomly linearized target DNA, then excised with flanking nucleotides to create small substitutions or deletions. For example, a linker with two copies of the recognition sequence for the enzyme *SapI* (GCTCTTC) is used to create six base deletions (three bases on each end of the linker), or random two amino acid deletions, across the *speB* gene, as illustrated in Figure 9. Flanking bases are also randomized by filling the ends of the target sequence after linker excision, then inserting a second blunt end linker that includes a random sequence in place of the Ns. The second linker is then removed by digestion with *SapI* and the target sequence is ligated to generate substitutions.

To identify protease minus mutations, *E. coli* cells producing potential mutant proteins are first screened for protease activity on casein agar plates. Since secretion of cysteine protease to the periplasm is expected, it is possible that protease activity can be observed on plates. If this screening strategy is successful, then thousands of colonies are rapidly examined for functional mutations in cysteine protease. If cysteine protease must be completely secreted from E. coli to exhibit activity, then osmotic shockates of each presumptive mutant strain is assayed for

protease activity.

EXAMPLE 21

Passive immunization of mice

Passive immunization with rabbit antibody directed against purified denatured cysteine protease partially protects mice against challenge with live S. pyogenes (Figure 10). The ability of rabbit antibody raised against purified cysteine protease to protect mice challenged intraperitoneally with a lethal dose of live *S. pyogenes* was examined. The likelihood of demonstrating protection was minimized because (i) rabbit antibody was raised against denatured cysteine protease (not the native zymogen or protease forms), (ii) the challenge strain also expressed pyrogenic exotoxin A (SPEA), and (iii) the cysteine protease variant made by the challenge strain (SPEB4) differs from the cysteine protease variant against which the antiserum was raised (SPEB1) at two amino acids (Ala<->Val at position 111 and Ser<->Gly at position 308).

EXAMPLE 22

Mouse immunization - intranasal

Intranasal immunization experiments are conducted essentially as described by Bessen and Fischetti (1988). Briefly, outbred Swiss CD1 mice of the same gender, 4 to 5 weeks old at the onset of immunization are used. Groups of 24 mice are immunized intranasally (i.n.) with 0 or 20 to 100 μg of zymogen, mature, active protease or synthetic peptide. The mice are immunized once each on days 1, 3, and 5 are rested for 3 weeks and boosted i.n. with a single dose of antigen (20 or 40 g). The initial group of 20 immunized are randomized to two subgroups of 10 animals. Each subgroup is challenged with either the cysteine protease source strain (homologous challenge) or a strain expressing a distinct cysteine protease variant

(heterologous challenge). Mice are given 10 ul of the bacterial suspension per nostril at 10 days after the cysteine protease boost. The vaccine is delivered i.n to unanesthetized mice (10 ul per nostril) through a model 750 Hamilton syringe equipped with a repeating dispenser and blunt-end needle. Throats are swabbed beginning 24 hours after challenge and at 24- and 48-hour intervals thereafter until day 11. Additional throat cultures are taken on day 15. Throat swabs are cultured on blood agar plates overnight at 37°C in a CO_2 incubator and beta-hemolytic colonies are counted the following day.

EXAMPLE 23

Bacterial challenge (intranasal)

The initial group of 24 immunized mice is randomized to two subgroups of 12 animals. Each subgroup is then challenged with either the SPEB source strain (homologous challenge) or a strain expressing a distinct SPEB variant (heterologous challenge). Strains used for challenge are selected for resistance to 200 μg/ml of streptomycin to facilitate recovery after challenge and if necessary are serially passaged by repeated i.p. injections in mice to increase ability to colonize and infect mice. A single stock of each challenge organism expressing SPEB is prepared from an overnight culture, concentrated 10-fold and stored at -80°C. Stocks are diluted 1:500 and grown overnight at 37°C in BHI broth, diluted 1:20 in fresh growth medium and cultured to an O.D.650 of 0.5. The cells are harvested by centrifugation and suspended in saline to about 2.5 x 10^5 CFU/ml. Inasmuch as streptococci strains differ in ability to colonize mice intranasally, a challenge dose is used that reproducibly colonizes greater than 25% of a nonimmune mouse population. Animals are housed six per cage by cohort. Mice are given 10 μl of the bacterial suspension per nostril at 10 days after the cysteine protease boost. Throats are swabbed beginning 24 hours after challenge and at 24- to 48-hour intervals thereafter

until day 11. Additional throat cultures are taken on day 15. Throat swabs are cultured on blood agar plates with 200 μg/ml of streptomycin, cultured overnight at 37°C in a CO_2 incubator and beta-hemolytic colonies are counted the following day.

EXAMPLE 24

Immunization of mice - subcutaneous

Immunization experiments are conducted in 4 to 5 week old outbred, immunocompetent, hairless mice (strain Cr1:SKH1 (hrhr)Br; Charles River) of the same gender. These mice are used because lesion size and character is easily cored and animals do not need to be shaved. Groups of 24 mice are immunized subcutaneously (s.c.) with 0, 20, or 40 μg of zymogen or mature, active protease. The mice are immunized once each on days 1, 7, 14, and 21, rested for 3 weeks and boosted with a single dose of protease (20 or 50 μg). The mice are checked for seroconversion by a cysteine protease-specific ELISA.

EXAMPLE 25

Bacterial challenge - subcutaneous

Immunized mice are randomized to two groups of 12 animals. Each group is then challenged with either the cysteine protease source strain (homologous challenge) or a strain expressing a distinct cysteine protease variant (heterologous challenge). A single stock of each challenge organism expressing cysteine protease is prepared from an overnight culture and adjusted to 10^6 CFU/ml. Mice (housed six per cage by cohort) are given 100 μL of the bacterial suspension mixed with an equal volume of sterile detran beads. The animals are inoculated s.c. on the right flank with a tuberculin syringe. Bacterial dilutions are prepared at the time of challenge to determine the exact number of CFU used. Negative control animals

consist of a group of 12 mice sham immunizations. These mice are "challenged" with only sterile medium plus dextran beads.

EXAMPLE 26

Immunological assays

Saliva and serum are collected from all immunized and control mice. Whole saliva is collected by pilocarpine stimulation 920 μg/mouse, subcutaneous) and centrifuged at 15,000 x g for 20 minutes. The material is divided and protease inhibitors are added to one of the aliquots. Storage is at -80°C. Serum is collected by bleeding from the tail vein. Individual and pooled saliva and serum from mice assigned to each cohort, and control mice, are assayed for specific antibody to cysteine protease by ELISA.

EXAMPLE 27

Data analysis

Animals are weighed immediately prior to challenge and every 24 hours post-challenge. Abscess volumes and area of dermo-necrosis will be calculated, and a lesion-size curve determined. Mean lesion sizes are compared statistically between groups by analysis of variance (ANOVA).

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned,

as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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WHAT IS CLAIMED IS: